Spectroscopic Evidence for Site-Specific Binding of the Cationic Porphyrin TMAP to a DNA Three-way Junction†

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Abbreviations: TMAP : meso-tetrakis (para-N-trimethylaniliniumyl) porphine; MD (Molecular Dynamics); NOE (nuclear Overhauser effect); NOESY (two-dimensional NOE spectroscopy);

ABSTRACT

The sterically bulky cationic porphyrin, meso-tetrakis (para-N-trimethylaniliniumyl) porphine (TMAP), is unable to intercalate duplex DNA and thus less effectively photosensitizes duplex DNA damage than the intercalating porphyrin 5,10,15,20-tetrakis(N-methyl-4-pyridiniumyl)porphine (T4MPyP). TMAP does, however, effectively photosensitize DNA at three-way junction (3WJ) sites [Nussbaum et al. (1994) Photochem. Photobiol. 59, 512-28]. NMR, optical absorbance and circular dichroism spectroscopic studies and gel-electrophoresis experiments are reported showing that TMAP binds specifically at the 3WJ formed by the oligonucleotide 5'-CGT GCA CCC GCT TGC GGC GAC TTG TCG TTG TGC ACG-3'. Addition of the DNA causes large red-shifting and hypochromicity in the Soret absorption band of TMAP and induces a negative CD band in the same spectral region. No such changes were observed for a control oligonucleotide designed to form a hairpin loop-stem structure. 2D NOESY spectra acquired at 600 MHz for [TMAP]/[DNA] ratios of 0.5, 1.0, and 2.0, exhibited differential broadening of resonances assigned to DNA protons at the junction site. This data provides the first physical evidence for specific, intercalative-like binding of TMAP to DNA. The use of TMAP with 3WJ-forming oligonucleotides, can lead to the design of specific photo-activatable DNA-targeting agents.

INTRODUCTION

In previous work reported by this laboratory it was shown, using photochemical probing and gel electrophoresis, that the cationic porphyrin TMAP (Fig. 1A) binds to DNA three-way junctions (3WJ) (1). The data suggested, but did not prove, that this binding occurs specifically at the 3WJ, a unique binding mode for TMAP to DNA. Three-way (3WJ) and four -way junctions (4WJ) have been reported to provide specific binding sites for other positively charged planar drugs (1-5). For example, it was reported that the water-soluble cationic porphyrin tetrakis(4-N-methylpyridiniumul)porphine (T4MPyP) and its Cu(II) and Ni(II) derivatives bound preferentially in the neighborhood of branch points of the DNA 4WJ (5). Recent crystallographic analysis showed minor groove binding of Ni^{2+}-T4MPyP to A/T region of DNA duplex (6). However, T4MPyP also has the ability to intercalate at 5'-CG-3' sites of the DNA duplex (7-11) and thus less specific for junctions. Moreover, site-specific interaction of the antitumor antibiotic dynemicin has also been reported for branched DNA molecules (12).

High-field NMR (500, 600, and 750 MHz) has been applied to characterize the three-dimensional structures of DNA 3WJs (13-16). These studies showed that appropriately designed DNA oligonucleotide sequences incorporating unpaired bases at the junction site can fold into structures exhibiting specific stacking of two of the helical arms of the 3WJ (13-15). Further evidence for specific helical stacking in DNA 3WJs was provided by gel mobility shift studies employing 3WJs having arms of different lengths (17). Previously, we reported molecular modeling studies, which proposed that the bulky cationic porphyrin, TMAP, should be able to intercalate a DNA 3WJ junction between the two stacked helical arms (1). This is a unique binding mode for TMAP to DNA, since this porphyrin is too large to intercalate to B-type duplex DNA (18-20). Here we provide experimental data to evaluate and refine these models.

The interest in 3WJ structures as potential targets for anti-sense photosensitizers arises from the fact that these structures occur in single-stranded infectious DNAs, for example, at the ends of the single-stranded parvovirus genomes. Such structures are in fact essential for the DNA replication and packaging (21). Also, 3WJs occur as intermediates in replication of long, simple sequence repeats in DNA (22,23), which can provide targets for anti-sense agents. Moreover, it has been shown that covalently attached intercalators to oligonucleotides,which designed to form 3WJ, can stabilize the 3WJ, if the intercalator is attached near the junction site (24).

For this study we chose a 36-mer DNA oligonucleotide previously studied by NMR and shown to fold into a stable 3WJ (Fig. 1A) (15). The sequence at the junction site is identical to 3-strand samples studied in our laboratory (13,14). The 3WJ comprises two unpaired thymidine bases at the junction region. Two of the helical arms are capped with stable 5'-CTTG-3' hairpin loops (25). The presence of the two unpaired bases at the branch point significantly stabilizes DNA 3WJ molecules (26). By using a single oligonucleotide that incorporates two stable hairpin loops into the sequence, the thermal stability of the molecule was further increased. The T_m of the molecule is 20°C higher than 3WJ composed of 3 separate strands (Bo Li & N.B. Leontis, unpublished data). This work presents the first 2D NMR studies of a porphyrin binding to a DNA 3WJ.

MATERIALS AND METHODS

Sample preparation.

DNA oligonucleotides were obtained from Oligo's Etc. Inc. (Wilsonville, OR.) The molar extinction coefficients for the oligomers were calculated from nearest neighbor parameters (27).

The chloride form of TMAP was obtained from Mid-Century (Posen, IL). TMAP concentrations were determined using $\varepsilon(\lambda_{max}) = 4.16 \times 10^5\ M^{-1}\ cm^{-1}$ (28).

Native Gel Electrophoresis.

The DNA/Porphyrin samples were electrophoresed on 20% non-denaturing polyacrylamide gels in moderate ionic strength buffers (0.05 M NaCl, 5 mM $MgCl_2$, 89 mM Tris-base, 89 mM boric acid, pH 8.1, and 2 mM EDTA). DNA oligonucleotides (3 nanomoles) were dissolved in 15 µl of this buffer and annealed at 90°C. Porphyrins were added after annealing to obtain [TMAP]/[DNA] ratios in the range between 0.0 and 2.0. The reaction mixtures were incubated for 30 minutes in the dark before electrophoresis. For gel loading, 10 µL of the same buffer, containing also xylene cyanole FF and 50% glycerol, were added to the DNA samples. Gels were run for 15 hours in the dark at 5°C using an electric field of 10 V/cm and recirculation of the buffer. The gels were photographed (f 4.0, 6 seconds) using a hand-held, short-wavelength UV lamp (254nm) to shadow the DNA by absorbance on a fluorescent TLC plate. The gels were also photographed (f 4.0, 4 seconds) with a medium-wavelength UV transilluminator (Foto-Dyne) to visualize porphyrins by fluorescence.

Absorption and CD Spectroscopy.

Absorption spectra measurements were recorded using an HP8452A diode array spectrophotometer. All solutions were prepared in 20 mM phosphate buffer (pH 7.2) containing 0.05 M NaCl and 0.1 mM EDTA. The same buffer was used for the NMR experiments. Absorption titrations were conducted by adding microliter aliquots of a concentrated stock solution of DNA (4.47×10^{-4}M) to TMAP solutions (3.58×10^{-6}M) in a 10 mm UV quartz cuvette. The absorption spectrum of the porphyrin/DNA solution was recorded over the spectral range of 200-700 nm. Circular dichroism spectra were obtained in the same buffer using an

AVIV and Associates 62DS circular dichroism spectrometer (Lakewood, NJ) equipped with a thermoelectric temperature controller. The spectrometer was calibrated with an aqueous solution of (1S)-(+)-10-camphorsulfonic acid. The spectra were obtained by averaging 2 scans using a wavelength step of 1 nm. The titrations were performed in the same manner as for the absorption experiments. The spectra were recorded over the spectral range 300-600nm.

Sample Preparation for NMR Studies of the TMAP/3WJ Complex.

For NMR experiments, the lyophilized DNA was dissolved in 0.25 ml of 20 mM Phosphate buffer, 50 mM NaCl, and 0.1 mM EDTA to a concentration of ~1.7 mM. For spectra acquired in H_2O, 5% D_2O was added for spin lock. The DNA sample was titrated with porphyrin by removing it from the NMR tube and using it to dissolve lyophilized TMAP aliquots. 2D NMR spectra were acquired at TMAP/3WJ molar ratios of 0.5 and 1.0.

NMR Spectroscopy for the TMAP/3WJ Complex. All NMR experiments were carried out in small volume NMR tubes (SHIGEMI, Inc., Allison Park, PA) on a 600MHz Varian Inova NMR spectrometer. Water suppression was achieved by standard Varian water presaturation methods using a presaturation delay of 1.5 seconds. 2D spectra were acquired with a spectral width of 8000 Hz in both dimensions, 2k complex points in t_2, 350 t_1 increments, 32 scans per increment, a recycle delay of 5.0 s for samples in H_2O and 3.0 s for samples in D_2O, and 300 ms mixing time. The NOESY data were acquired in the phase-sensitive mode with time-proportional phase incrementation (TPPI). NMR data were processed using Felix95 software (Biosym/MSI, San Diego). Data in the D1 and D2 dimensions were zero-filled to 2048 and 1024 real points, respectively, and apodized by multiplication with a 90° shifted squared sinebell function.

Molecular Modeling.

To obtain molecular mechanics parameters for TMAP *ab initio* quantum mechanical calculations were carried out using Hyper Chem4.5 (Hypercube, Inc., FA). TMAP was modeled as two fragments, one of which comprised the porphine nucleus and the other the para-N-trimethylaniliniumyl pendant groups attached to the meso positions of the porphine. Allyl groups were attached (at C2) to the meso positions of the porphine to model the sp^2-hybridized carbon of the pendant groups, and allyl groups were attached to the para positions of the 4-N-methyl-pyridiniumyl pendant groups to model the sp^2 meso-carbons of the porphine. The geometries were optimized with Hartree-Fock *ab initio* methods at the 3-21G basis set level. Atom-centered charges were obtained by fitting the electrostatic potential calculated *ab initio* with the 6-311G* basis set from coordinates optimized at the 3-21G level using the routines provided by the HyperChem program. AMBER atom types were assigned according to published guidelines and torsional and bond stretching constants were scaled according to bond length (29). AMBER topology and parameter files for TMAP and for the 3WJ were generated using the xLeap module of AMBER5.0 (30) with parm98 provided by Tom Cheatham. The TMAP molecule was docked at the 3WJ so that it was fully inserted between the basepairs of Helices I and II. To neutralize the negative charges of the phosphates, 30 Na^+ ions were distributed around the porphyrin/DNA complex. A cutoff distance of 12 Å was used for calculating Lennard-Jones interactions throughout. The SHAKE option was used to constrain all bonds involving hydrogen. The presence of a high dielectric solvent was simulated using distance dependent dielectric constant, $\varepsilon=4r_{ij}$, where r_{ij} is the distance separating the pair of atoms (31). The use of $\varepsilon=4r_{ij}$ provides an optimal fit of experimental and calculated values for the parameters defining DNA structure (32).

The structure was first energy minimized by the steepest descent method to a gradient < 1kcal/Å-mol. This was followed by conjugate gradient minimization to a gradient < 0.01kcal/Å-mol. To retain structural integrity, the distances between the atoms participating in Watson-Crick hydrogen bonding were constrained (2.81Å-3.01Å for GO6-CN4, 2.85Å-3.05Å for GN1-CN3, 2.76Å-2.96Å for GN2-CO2, 2.76Å-2.92Å for AN1-TN3 and 2.85Å-3.05Å for AN6-TO4) (33,34). Simulated annealing (35) was employed to escape local minima obtained by energy minimization and perform conformational search for the TMAP/DNA complex. This procedure has been used to model modified DNA structures (36-38), to optimize the structures of carcinogen-DNA complexes (39), and to study folding processes in RNA (40).

Simulated annealing was carried out gently enough to allow the DNA/drug complex to explore the conformational region without escaping the input conformational class or becoming grossly distorted. In addition to hydrogen bond constraints, a 1kcal/mol force constant was employed to restrain the movement of purine and pyrimidine rings to mimic the stacking interactions between the bases forming the helical arms of the 3WJ. This value was chosen based on thermodynamic measurements of the stacking energies of single bases on the 3'-ends of helices (41,42). To allow flexibility at the junction site, we did not apply any restraints to the basepairs flanking the junction site or to any of the unpaired thymidines at the junction or in the hairpin loops. Following simulated annealing, a final 1000 steps of conjugate gradient minimization were carried out without any constraints. To optimize the simulated annealing protocol we arrayed several parameters such as annealing temperature (T), force constant for the hydrogen restraints (k), annealing time (t), length of heating (t_H) and cooling phases (t_c). A total of 30 simulated annealing runs were performed on the 3WJ/TMAP complex. At annealing temperatures above 500K, the structures were significantly disrupted, and therefore not

considered. For structures obtained at annealing temperature below 500K, the total AMBER and van der Waals energies were calculated and compared. The lowest values were obtained for the following protocol: T= 400K, k=100 kcal/mol, t=25 ps, t_H = 8 ps and t_c= 15 ps, time step = 1fs. This protocol was also employed for complexes of TMAP and of T4MPyP with the DNA duplex $[d(CCCGGG)]_2$. Previously calculated modeling parameters for T4MPyP were used (11). Each porphyrin was docked between the central C=G basepairs of the duplex so that two of the 4-N-methyl-pyridiniumyl groups were located in the minor groove and the other two were located in the major groove of the duplex. It was shown previously that T4MPyP intercalates duplex DNA in this symmetrical orientation in solution (11).

RESULTS

Interaction of TMAP and 3WJ by Native Gel Electrophoresis.

The sequences and secondary structures of the 3WJ- and hairpin-forming oligonucleotides used in these studies are shown in Figures 1B and 1C. The sequence of the DNA mini-hairpin (MH) corresponds to one of the helical arm of the 3WJ. Previous studies showed that TMAP does not intercalate in duplex DNA (1). Thus, MH DNA was synthesized to test whether TMAP binds specifically to the stable TT hairpin loop used to terminate two of the helical arms in the 3WJ.

Native gel electrophoresis was used to monitor the binding of TMAP to DNA samples, as described above and in previous work (1). Results are shown in Figure 2. The molar ratio of the TMAP/DNA in lanes 1 to 5 in Fig. 2 increases from 0.0 to 2.0. Panel A in Fig. 2 shows the position of the DNA in the TMAP/DNA complex after electrophoresis, as visualized by UV-shadowing on a fluorescent TLC plate. Panel B shows the position of the DNA-bound porphyrin

as visualized by porphyrin fluorescence. The UV-shadowed band and the porphyrin fluorescence coincide exactly, indicating that the cationic TMAP porphyrin binds tightly to the DNA 3WJ and co-migrates with it toward the positive pole. Binding of TMAP induces a slight electrophoretic mobility retardation in the DNA/porphyrin complex (lane 5), compared to the DNA alone (lane 1). This is consistent with the smaller (net charge)/mass ratio of the porphyrin-DNA complex. The broadening of the fluorescent band, observed in panel B, indicates that a fraction of the porphyrin dissociated from the 3WJ DNA during the course of the experiment (15 hours) and migrated back toward the negative electrode. However, most of the porphyrin remained bound to the 3WJ throughout electrophoresis. In the case of the hairpin forming DNA (MH DNA), the porphyrin separated from the DNA and its fluorescence was only observed at the top of the electrophoresis wells (Figure 2B, left side of the gel), indicating lack of the affinity of TMAP toward stem loop of the 3WJ (MH DNA).

Optical Absorbance and Induced Circular Dichroism studies.

Figure 3A shows absorption spectra obtained by addition of concentrated 3WJ to a fixed amount of TMAP porphyrin (initially 3.6×10^{-6} M). Spectra were corrected for volume changes due to the addition of DNA, which in all cases was less than 5%. In Figure 3A, the ratio [DNA]/[TMAP] ranges from 0 to 2.0. The DNA 3WJ induces a 10 nm red shift in the Soret band, from λ_{max} = 412 nm for the free porphyrin to λ_{max} = 422 nm for porphyrin bound to 3WJ DNA, and significant hypochromicity of the porphyrin absorption (56% at 422 nm and 33% at the respective values of λ_{max}). However, a distinct isosbestic point was not observed.

In the absence of 3WJ, no CD signal was observed for the optically-inactive TMAP porphyrin. At [DNA]/[TMAP] = 0.25, a negative induced CD band with λ_{max} = 424 nm appeared (Fig. 3B). As the [DNA]/[TMAP] ratio was increased, the negative intensity of the

induced CD band also increased. Red shifts in λ_{max} and substantial hypochromicities in the porphyrin absorption spectrum, accompanied by large, negative induced CD signals, have been associated with intercalative binding to DNA (43-46).

To determine whether the effects on TMAP absorption and CD spectra are specifically due to the 3WJ, we carried out experiments on a stem-loop DNA sample comprising the TT minihairpin, extended by a 12 basepair duplex, "EX" DNA (Fig. 1D). Figure 4 shows spectra obtained by the addition of EX DNA to a fixed amount of TMAP (initially 3.6×10^{-6} M). The ratio [DNA]/[TMAP] ranges from 0 to 3.0. The absence of a red shift in the absorption spectra (Fig. 4A) and of negative CD bands (Fig. 4B) corroborate the gel electrophoresis results indicating that TMAP does not bind tightly to the TT minihairpin loop of the 3WJ or to the extended double helical surface of the stem. Thus, it appears that high affinity TMAP binding to the 3WJ DNA likely occurs at the junction itself. Two-dimensional NMR studies were carried out to investigate the details of the interaction.

NMR spectroscopy: Proton Assignment of the 3WJ.

Complete proton assignments for the 3WJ were needed to determine the binding mode between the 3WJ and the TMAP. As a starting point, the published assignments of the non-exchangeable protons for the 3WJ were used (15). As numerous small differences in chemical shifts were noted with the published data, due to unavoidable differences in sample preparation and data acquisition, we repeated the entire resonance assignment procedure, using sequential methods of NOESY assignments (47). The chemical shift table (Table S1), the thymidine-methyl to aromatic (Figure S1) and H1' to the H6/H8 (Figure S2) regions of NOESY spectra can be found in supplemental material section of this paper. Here, we briefly reviewed the important

points of the assignments to make the reader familiar with the 3WJ DNA spectra, as it is crucial to the interpretation of the porphyrin binding experiments.

Intense H6 to methyl crosspeaks were observed for nine Thymidine residues (four thymidines of the two hairpin loops (T23/T22 and T13/T12), the three thymidines that occur in helical regions (T25, T31 and T3), and the two unpaired thymidines at the junction site (T28 and T29)) in the 2D NOESY spectrum of 3WJ DNA (see Fig. S1, Supplemental Material). The two most downfield crosspeaks in the methyl region (around 2.0 ppm) correspond to T12Me-H6 and T22Me-H6 in the hairpin loops (1.98/7.82 ppm for the T22 and 2.04/7.90 ppm for the T12). The crosspeaks at 1.81 ppm/7.44 ppm and 1.65 ppm/7.49 ppm were assigned to T28Me-H6 and T29Me-H6, respectively. The remaining methyl resonances occur between 1.5 and 1.25 ppm, where thymidine resonances in B-type DNA double helices are expected (47). We observed NOEs between the C21-H6 and T23-Me and between C11-H6 and T13-Me, indicating that the T23 base is partly stacked on C21 and that T13 is partly stacked on C11 in the hairpin loops in 3WJ, as also occurs for the isolated hairpin loops (25). These NOEs have not been published by Overmars *et. al* and reported for the first time for the 3WJ DNA (see Figure S1, Supplemental Material). Moreover, NOEs were observed between T13-Me and C11H1'/H5 as well as between T23-Me and C21H1'/H5, confirming the stacking between the second thymidine and the Cytosine base in each hairpin loops of the 3WJ. The long-range NOE between the methyl resonances of unpaired T28 at the junction and H6 of C7, flanking the junction at the end of helix III, was also observed (see Fig. S1, Supplemental Material). This and other NOEs between T28 and C7 indicate that these two bases are stacked.

Intra- and inter-residue NOEs characteristic for B-type DNA helices are observed for the helical arms (see Fig. S2, Supplemental Material). The sequential NOE path extends from C1 to

C7, but is interrupted between C7 and C8, indicating that these bases are probably not stacked. Instead, as noted by Overmars *et. al,* NOEs characteristic of B-form DNA were observed between G17 and C18 (labeled in Fig. S2, Supplemental Material) and long-range NOEs were observed between C8 and G27 (for example, the G27-H8/C8-H5 crosspeak indicated in Fig. 2S, supplemental material). G17=C8 is the basepair on the end of Helix II flanking the junction. C18=G27 is the basepair on the end of Helix III flanking the junction. These data are consistent with stacking of Helices II and III at the junction as indicated in Figure 1B and previously concluded (15).

Several NOEs are observed between T28 and T29 indicating that these bases are stacked. As already noted, T28 is stacked on C7. Very weak NOEs were observed between G27 and unpaired T28 indicating little or no stacking. No NOEs were observed between the unpaired T29 and G30, which pairs to C7 in Helix III flanking the junction. Continuous sequential NOEs are, however, observed from G30 to G36 and from C1 to C7.

On the basis of observed NOEs, we built a heuristic 3D model using the MANIP program (48). The model was constructed by stacking B-form Helices II and III and attaching Helix III (with the unpaired T28 and T29) at the break point between nucleotides C8 and G27. T28 and T29 were stacked above C7. A tight turn was modeled between T29 and G30 resulting in strand reversal between these nucleotides. Thus C7-T28-T29-G30 resemble a hairpin loop, except that C7 and T28 are not covalently linked. This model is qualitatively consistent with all the NMR data. Refinement was carried out with MANIP refinement tool NUCLIN-NUCLSQ, using basepairing constraints and restraints imposed by covalent geometry, stereochemistry and van der Waals contacts.

NMR studies of the TMAP/3WJ complex.

NMR spectra were acquired at [TMAP]/[3WJ] ratios ranging from 0 to 2.0. One-dimensional spectra acquired for [TMAP]/[3WJ] = 0, 0.5, 1.0, and 2.0 are shown in Figure 5. No new DNA resonances were observed upon addition of TMAP to the DNA duplex. The addition of TMAP was accompanied by specific line-broadening of DNA resonances, even at the lowest ratio of porphyrin to DNA used ([TMAP]/[3WJ] = 0.25, data not shown). For example, at 7˚C the average linewidths of the methyl resonances were found to increase from 6 Hz in the absence of porphyrin to 15 Hz ([TMAP]/[3WJ] = 0.5). Such broadening is likely due to slower tumbling of the complex relative to DNA 3WJ and, more importantly, exchange of drug between DNA and solution, or between competing sites on the 3WJ. The intense singlet resonating at 3.86 ppm was assigned to the CH_3 groups of the four trimethylaniliniumyl groups of the TMAP molecule bound to DNA, and is indicated by an arrow in the spectra shown in Figure 5. Certain DNA resonances in the methyl region of the 1D spectrum (shown in detail in Fig. 6) were significantly broadened in the presence of TMAP. The methyl region of the 1D spectra taken in absence of TMAP is shown in Fig. 6A. The methyl resonances of the unpaired T28 and T29 at the junction were most affected, even at the smallest ratio, [TMAP]/[3WJ] = 0.5 (Fig. 6B). At [TMAP]/[3WJ] = 1.0 these two peaks were almost undetectable (Fig. 6C). However, the rest of the methyl peaks remained distinct. At the highest [TMAP]/[3WJ] ratio studied (2:1), the T29 methyl peak completely disappeared (Fig. 6D).

NOESY experiments were carried out on the porphyrin/DNA complex at [TMAP]/[3WJ] ratios of 0.5 and 1.0. The aromatic regions of NOESY spectra for the DNA alone and for TMAP/DNA complexes at molar ratios of 0.5 and 1.0 are compared in Figure 7. Although no NOEs could be observed for the free porphyrin in solution on account of its intermediate

molecular weight, intra-porphyrin NOESY crosspeaks were observed in the presence of 3WJ DNA (Fig. 7B and C). In the free TMAP, the β-pyrrole protons resonate at 9.1 ppm, the meta protons at 8.45 ppm, the ortho protons at 8.3 ppm and the methyl protons at 4.00 ppm. In the presence of the 3WJ DNA, the porphyrin resonances are shifted upfield, to 8.47 ppm, 8.30 ppm, 8.16 ppm and at 3.86 ppm (most intense peak). At high TMAP/3WJ ratios, we observed NOEs between the 8.30 and 8.16 ppm TMAP resonances and between these same resonances and the protons resonating at 3.85 ppm, which can be assigned to the TMAP methyl protons. Thus, the NOE between the resonances at 8.30 and 8.16 ppm can be reasonably assigned to the meta and ortho protons of the trimethylaniliniumyl groups. Based on the chemical shifts for the free porphyrin (49), one expects the ortho protons to resonate upfield of the meta protons. Therefore, the resonance at 8.30 ppm can be assigned to the meta proton and the resonance at 8.16 ppm to the ortho proton. By a process of elimination, the resonance at lower field, 8.47 ppm, which does not give NOEs to the methyl resonances of the trimethylaniliniumyl groups, was assigned to the β-pyrrole protons (Fig. 7C). In summary, the TMAP resonances in the 3WJ complex are: 8.47ppm for the β-pyrrole protons, 8.30 ppm for meta, 8.16 ppm for ortho, and 3.86ppm for CH_3. Thus, the largest chemical shift induced by DNA binding is -0.63 ppm for the β-pyrrole protons. The methyl and aromatic ring protons show the same shifts, about -0.14 ppm. These large, negative (upfield) chemical shifts are consistent with intercalative binding modes for the porphyrin in the 3WJ (11). Interestingly, the chemical shift of the β-pyrrole protons of TMAP bound to 3WJ is considerably larger than that observed for the β-pyrrole of T4MPyP intercalated into duplex DNA (-0.67 vs. –0.22 ppm) but also in the upfield direction (11), consistent with intercalative-like binding.

The aromatic-H1' regions of 2D NOESY spectra of the 3WJ in the absence and presence of TMAP ([P]/[DNA] = 1:2) are compared in Figs. 8A and 8B. These spectra were both acquired at 7°C (mixing time =300 ms) in H_2O. Only selected NOEs are labeled for clarity. NOE connectivities for resonances belonging to residues near the junction are traced with dotted lines. Connectivities for the hairpin loop resonances are indicated with solid lines. Fig. 9 shows the aromatic-methyl region for the TMAP/DNA complex, also at a 1:2 molar ratio. DNA resonance assignments were obtained from these spectra. For samples containing higher ratios of [TMAP]/[3WJ], spectral broadening did not allow us to follow sequential connectivities.

Comparing the NOESY spectra in Figs. 8A (no TMAP) and 8B ([TMAP]/[3WJ] = 1:2), porphyrin induced broadening is evident for almost all of the DNA NOESY crosspeaks. Moreover, addition of TMAP led to the disappearance of all crosspeaks involving exchangeable cytosine amino proton resonances (for example the C21 amino/H5 NOE, labeled in Figure 8A and absent in Figure 8B).

Selective broadening or even disappearance of a subset of non-exchangeable resonances is observed. In the cytosine H5-H6 region, the C7 and C18 crosspeaks are most affected. No conclusion can be drawn regarding C8, which together with C7 and C18 directly flanks the junction site, because its H5-H6 crosspeak overlaps with the T13 H6-H1' crosspeak. Other well-resolved cytosine H5-H6 crosspeaks, including C15 (Helix II), C21 (Helix III), and C35 (Helix I) are much less affected. These cytosines are further removed from the junction. In addition to broadening, small chemical shift changes are observed for the H5 and H6 resonances of C7 and C18, which are not observed for other cytosines. The other NOEs involving these residues are also selectively affected by TMAP, including A6-H8/C7-H5, C7-H6/C7-H1', C18-H6/C18-H1', and C18-H6/G17-H1'. The C7-H6/A6-H1' and the C18-H5/G17-H8 NOEs completely

disappear. The G17 H8-H1' and G17-H8/G16-H1' NOEs are also significantly broadened and shifted so that they overlap the C1 H5-H6 and C1 H6-H1' crosspeaks.

The differentially affected NOEs are labeled by red letters in Figure 8B, showing that they all belong either to C18, C7, G17, G27, or to T31. The T31 H6-H1' crosspeak (which is labeled "T31" in Figure 8B) was much less affected by the addition of TMAP than the T31-H6/G30-H1', which is the peak labeled "T31*" in Figure 8B. G30-H1' is closer to the junction than T31-H1'. Certain NOEs were completely eliminated by the presence of TMAP. NOEs most affected by addition of TMAP are summarized in Table 1. Almost all NOEs involving one or more proton adjacent to the junction site were strongly affected and showed significant broadening (labeled "broadened" in Table 1). In Figures 8A and 8B, we also provide sequential assignment for the minihairpin loops (solid black lines). It is apparent that the NOEs in the minihairpin loops of the 3WJ are much less affected by the addition of the porphyrin. The observation of the intermolecular NOEs C21-H6/T23-Me and C11-H6/T13-Me at 1:2 [P]/[3WJ] ratio indicates that T13 remains stacked on C11 in the minihairpin loop (Fig. 9). The observation of the differential changes in the 2D spectra of the 3WJ at [P]/[3WJ] = 0.5 supports our conclusion made from the 1D spectra analysis, that the TMAP strongly affects resonances primarily at the junction site. Thus, the observed selective broadening of NOEs serves to indicate the most likely location of TMAP when bound to the DNA. We were not able to identify all NOEs involving T28-Me and C7-H6 seen in the absence of porphyrin in the TMAP/3WJ complex due to the spectral broadening. However, the observation of the T28-Me/C7-H6 (Fig. 9) indicates that T28 remains stacked on C7 in the presence of TMAP, which is a strong evidence for 3WJ intergrity.

No intermolecular NOEs were found between TMAP and the 3WJ protons. Moreover, only one set of DNA resonances is observed upon TMAP addition. Small chemical shift changes (less than 0.1 ppm) are observed for most of the resonances that also show selective broadening in the NOESY spectra. One of the largest shifts (0.08 ppm) is observed for T29-H1' and larger than for T28-H1' (0.05 ppm). The observation of a single set of resonances indicates intermediate to fast exchange kinetics for the TMAP/3WJ interaction. DNA resonances of protons located farther from the TMAP binding site are expected to be less affected by porphyrin binding and thus the change in chemical shift ($\Delta\delta$) is smaller than the exchange rate. This leads to fast exchange and sharper lines than for resonances of protons close to the binding site, for which $\Delta\delta$ is comparable in magnetic to the exchange rate, leading to intermediate exchange and greater resonance broadening. 2D NMR data combined with the observation of the negative intensity of the CD signal from the TMAP/3WJ complex allows us to propose that the interaction between TMAP to 3WJ can be characterize as intercalative binding. However, we would like to point out that the existence of the minor population of the non-intercalative binding to the 3WJ can not be rulled out and might be accounted for the lack of the distinct isosbestic point in our absoprtion spectra.

Molecular Modeling.

Intercalation would be a novel binding mode for the TMAP porphyrin, since this molecule is known not to intercalate into the DNA duplex (18-20). In this work, we decided to evaluate the possibility of intercalative binding for the TMAP to the 3WJ using molecular modeling. In Figure 10 (left panel), the model of TMAP intercalated in the 3WJ between the C8=G17 and the G27=C18 basepairs at the junction site is shown. The right panel of Figure 10 shows a model of

TMAP intercalated between the central C=G and G=C basepairs in the model DNA duplex $[d(CCCGGG)]_2$. This model was constructed to serve as a point of reference, as TMAP has not been observed to intercalate duplex DNA. Both models were constructed by hand-docking TMAP using MANIP followed by the simulated annealing protocol described in the Methods. In the model of the TMAP intercalated in the duplex, the bulky tri-methyl groups of TMAP prevent this porphyrin from intercalating optimally between the bases in the duplex. In the case of the 3WJ, however, the extra flexibility in the backbone of the DNA resulting from the presence of the junction site, especially the two unpaired thymidines, allows better accommodation of TMAP. AMBER energies for these complexes were calculated and tabulated in Table 2A**,** broken down by energy terms. Additionally, energies are reported for the T4MPyP/duplex DNA complex. In previous work, this porphyrin was shown, using 2D NMR and Molecular Modeling, to intercalate duplex DNA (10). For each energy-minimized complex, the energies of the porphyrin alone and of the DNA alone were calculated, without any further energy minimization. The AMBER interaction energy between porphyrin and DNA was then calculated as the difference between the energy of the complex and the sum of the energies of the porphyrin alone and of the DNA alone. For the TMAP/3WJ complex, this gave an interaction energy of –360 kcal/mol, while for the TMAP/duplex this gave -188 kcal/mol, as compared to –230 kcal/mol for the T4MPyP/duplex. The breakdown of energies shows that the larger (more negative) interaction energy obtained for TMAP/3WJ compared to TMAP/duplex is attributable to significantly greater van der Waals interactions (-87 vs. –32 kcal/mol) and more favorable electrostatic interactions (-269 vs. –155 kcal/mol). The more favorable T4MPyP/duplex interactions relative to TMAP/duplex are attributable only to more favorable van der Waals interactions (-75 vs. –32 kcal/mol), as the electrostatic interactions are comparable.

To examine the degree of distortion of the porphyrins themselves that is induced by the interaction with each DNA, we calculated the difference between the energy of each porphyrin in its bound conformation and an energy-minimized conformation. These data are shown in Table 2B. The intra-molecular energy of T4MPyP bound to the DNA duplex is only 5 kcal/mol greater than that of energy-minimized T4MPyP. The energy difference is largely due to a small increase in dihedral energy upon binding to DNA. By contrast, the dihedral energy term for TMAP bound either to the 3WJ or to the DNA duplex is quite large (66 vs. 74 kcal/mol). Nonetheless, the energy of TMAP is about 9 kcal/mol lower when bound to the 3WJ than when bound to the duplex and this is largely due to the difference in the dihedral energy term. Thus, TMAP suffers less dihedral distortion when bound to the 3WJ.

A comparison of the effect of porphyrin binding on the conformation of the DNA could be made for the TMAP/duplex and the T4MPyP/duplex (Table 2C). The energy of the duplex alone is significantly lower when complexed to T4MPyP (–809 kcal/mol) than when complexed to TMAP (-664.0 kcal/mol). The difference is 145 kcal/mol and is due to more favorable intramolecular van der Waals and electrostatic interactions in the DNA when it is bound to T4MPyP .

These calculations show that in the TMAP/3WJ complex, more favorable binding interactions occur and fewer unfavorable interactions are induced in either the porphyrin or the DNA than in the case of the TMAP/duplex intercalation complex, which in fact is not observed. Thus, an energetically reasonable model involving TMAP intercalation at the junction can be constructed, consistent with the spectroscopic data.

DISCUSSION

The spectroscopic data indicate that binding of 3WJ DNA induces a large bathochromic shift and hypochromicity in the Soret absorption band of TMAP, as well as negative circular dichroism, which is a distinct characteristic of intercalative binding. Furthermore, TMAP binding causes differential exchange broadening of NMR resonances assigned to residues located at the three-way junction. Resonances assigned to all residues flanking the junction were affected, including the unpaired T28 and T29 nucleotides. Some of the largest downfield chemical shift changes were observed for T29, which is located at the edge of the porphyrin nucleus in the molecular model (Figure 10). Only one set of resonances is observed for the 3WJ/TMAP complex at a 1:2 molar ratio, indicating intermediate to fast exchange. Thus, insertion and dissociation of TMAP from the junction is quite facile, in spite of the size and bulkiness of the TMAP pendant groups. Differential broadening and shifting is only observed for resonances assigned to the junction region of the DNA. Resonances assigned to hairpin loops were not affected in the same way by the presence of the porphyrin. This supports the conclusion made from the gel electrophoresis experiment, that TMAP does not bind to the hairpin loop motifs. In conclusion, the model in which TMAP is intercalatively inserted at the junction is reasonable on energetic grounds and is consistent with the spectroscopic data. This binding is stabilized by electrostatic interactions with multiple phosphate groups at the junction and by stacking with at least two of the helices. Our data provides the first evidence for intercalative binding of TMAP to a nucleic acid. However, the lack of the specific intra-molecular NOEs between TMAP and 3WJ, which can be due to the nature of exchange, makes it difficult to conclude that intercalation is the only possible interaction mode between this two molecules. Previously, the observation of intra-molecular NOEs between the T4MPyP and DNA duplex allowed us to conclude that this porphyrin intercalates to 5'-CG-3' step in DNA duplex

(11). Moreover, the absence of the distinct isosbestic point on the absorption spectra, allows us to suggest that minor population of site-specific non-intrecalative binding modes may also occur for the TMAP/3WJ complex.

The specificity for binding to junctions suggests that TMAP can be targeted to such structures much more specifically than other cationic porphyrins, such as T4MPyP, which also bind to duplexes. By combining the use of highly photoactive porphyrins and 3WJ-forming oligonucleotides, more specific and potent photo-activatable anti-sense agents can be designed. Moreover, the proposed model also accounts for the highly specific photosensitization of DNA three-way junctions by TMAP observed in previous work.

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REFERENCES

1. Nussbaum, J. M., Newport, M. E., Mackie, M. and Leontis, N. B. (1994) Structure-specific binding and photosensitized cleavage of branched DNA three-way junction complexes by cationic porphyrins. *Photochem. Photobiol.*, **59**(5)**,** 515-28.

2. Seeman, N. C. and Kallenbach, N. R. (1994) Dna Branched Junctions. *Ann. Rev. Biophys. Biomol. Struct.*, **23,** 53-86.

3. Guo, Q., Seeman, C. and Kallenbach, N. R. (1989) Site-specific interaction of intercalating drugs with a branched DNA molecule. *Biochemistry*, **28**(6)**,** 2355-2359.

4. Guo, Q., Lu, M., Seeman, N. C. and Kallenbach, N. R. (1990) Drug Binding by Branched DNA Molecules: Analysis by Chemical Footprinting of Intercalation into an Immobile Junction. *Biochemistry*, **29,** 570-578.

5. Lu, M., Guo, Q., Seeman, N. C. and Kallenbach, N. R. (1990) Drug Binding By Branched Dna - Selective Interaction of the Dye Stains-All With an Immobile Junction. *Biochemistry*, **29**(13)**,** 3407-3412.

6. Bennett, M., Krah, A., Wien, F., Garman, E., McKenna, R., Sanderson, M. and Neidle, S. (2000) A DNA-porphyrin minor-groove complex at atomic resolution: The structural consequences of porphyrin ruffling. *Proc. Natl Acad. Sci. USA*, **97**(17)**,** 9476-9481.

7. Marzilli, L. G., Banville, D. L., Zon, G. and Wilson, W. D. (1986) Pronounced 1H and 31P NMR spectral changes on meso-tetrakis(N-methylpyridinium-4-yl)porphyrin binding to poly[d(G-C)].poly[d(G-C)] and to three tetradecaoligodeoxyribonucleotides: Evidence for symmetric, selective binding to 5'CG3' sequences. *J. Am. Chem. Soc.*, **108,** 4188-4192.

8. Ford, K. G., Pearl, H. G. and Neidle, S. (1987) Molecular modeling of the interaction of tetra-(4-N-methylpyridyl) porphin with TA and CG sites on DNA. *Nucleic Acids Res.*, **15**(16)**,** 6553-6562.

9. Ford, K., Fox, K. R., Neidle, S. and Waring, M. J. (1987) DNA sequence preference for an intercalating porphyrin compound revealed by footprinting. *Nucleic Acids Res,*, **15,** 2221-2234.

10. Lipscomb, L. A., Zhou, F. X., Presnell, S. R., Woo, R. J., Peek, M. E., Plaskon, R. R. and Williams, L. D. (1996) Structure of DNA-porphyrin complex. *Biochemistry*, **35,** 2818-23.

11. Guliaev, A. B. and Leontis, N. B. (1999) The Cationic Porphyrin T4MPyP4+ Fully Intercalates at 5'-CG-3' Steps of Duplex DNA in Solution. *Biochemistry*, **38**(47)**,** 15425-15437.

12. Lu, M., Guo, Q. and Kallenbach, N. R. (1991) Site-Specific Interaction of the Antitumor Antibiotic Dynemicin With Branched Dna Molecules. *J. Biomol. Struc. Dyn.*, **9**(2)**,** 271-283.

13. Leontis, N. B., Hills, M. T., Piotto, M., Malhotra, A., Nussbaum, J. and Gorenstein, D. G. (1993) A model for the solution structure of a branched, three-strand DNA complex. *J Biomol. Struc. Dyn.*, **11**(2)**,** 215-23.

14. Leontis, N. B., Hills, M. T., Piotto, M., Ouporov, I. V., Malhotra, A. and Gorenstein, D. G. (1995) Helical stacking in DNA three-way junctions containing two unpaired pyrimidines: proton NMR studies. *Biophys. J.*, **68**(1)**,** 251-65.

15. Overmars, F. J., Pikkemaat, J. A., van den Elst, H., van Boom, J. H. and Altona, C. (1996) NMR studies of DNA three-way junctions containing two unpaired thymidine bases: the influence of the sequence at the junction on the stability of the stacking conformers. *J. Mol. Biol.*, **255**(5)**,** 702-13.

16. Rosen, M. A. and Patel, D. J. (1993) Conformational differences between bulged pyrimidines (C-C) and purines (A-A, I-I) at the branch point of three-stranded DNA junctions. *Biochemistry*, **32**(26)**,** 6563-75.

17. Welch, J. B., Walter, F. and Lilley, D. M. (1995) Two inequivalent folding isomers of the three-way DNA junction with unpaired bases: sequence-dependence of the folded conformation. *J. Mol. Biol.*, **251**(4)**,** 507-19.

18. Carvlin, M. J., Datta-Gupta, N. and Fiel, R. J. (1982) Circular dichroism spectroscopy of a cationic prophyrin bound to DNA. *Biochem. Biophys. Res. Comm.*, **108,** 66-73.

19. Carvlin, M. J. and Fiel, R. J. (1983) Intercalative and nonintercalative binding of large cationic porphyrin ligands to calf thymus DNA. *Nucleic Acids Res.*, **11**(17)**,** 6121-39.

20. Fiel, R. J. (1989) Porphyrin-Nucleic Acid Interactions: A Review. *J. Biomol. Struc. Dyn.*, **6,** 1259-1275.

21. Corsini, J., Afanasiev, B., Maxwell, I. H. and Carlson, J. O. (1996) In Maramorosch, K. F. A. M., and Shatkin, A. J. (eds.), Advances in Virus Research, Vol. 47. Academic Press, Inc., San Diego, California, USA; London, England, UK, pp. 303-351.

22. Wells, R. D. (1996) Molecular basis of genetic instability of triplet repeats. *J. Biol. Chem.*, **271**(6)**,** 2875-2878.

23. Zhao, Y., Cheng, W., Gibb, C. L. D., Gupta, G. and Kallenbach, N. R. (1996) HMG box proteins interact with multiple tandemly repeated (GCC)n.(GGC)m DNA sequences. *J. Biomol. Struc. Dyn.*, **14**(2)**,** 235-238.

24. Abdel-Rahman, A. A. H., Ali, O. M. and Pedersen, E. B. (1996) Insertion of 5-methyl-N-4-(1-pyrenylmethyl)cytidine into DNA. Duplex, three-way junction and triplex stabilities. *Tetrahedron*, **52**(48)**,** 15311-15324.

25. Blommers, M. J., Walters, J. A., Haasnoot, C. A., Aelen, J. M., van der Marel, G. A., van Boom, J. H. and Hilbers, C. W. (1989) Effects of base sequence on the loop folding in DNA hairpins. *Biochemistry*, **28**(18)**,** 7491-8.

26. Leontis, N. B., Kwok, W. and Newman, J. S. (1991) Stability and structure of three-way DNA junctions containing unpaired nucleotides. *Nucleic Acids Res.*, **19**(4)**,** 759-66.

27. Puglisi, J. D. and Tinoco Jr, I. (1989) Absorbace melting curves of RNA. *Methods in Enzymology*, **San Diego: Academic Press,** 413-475.

28. Kirshnamurthy, M. (1977) Synthesis and characterization of new water soluble porphyrin. *Indian J. Chem. Sect. B*, **15B,** 385-388.

29. Cornell, W. D., Kollman, P. A. and Ferguson, D. M. (1995) A second Genertaion Force Field for the Simulation of proteins, Nucleic Acids and Ogranic Molecules. *J. Am. Chem. Soc.* , **117,** 5179-5191.

30. Case, D. A., Pearlman, D. A., Caldwell, J. W., Cheatham, T. E., Ross, W. S., Simmerling, C. L., Darden, T. A., Merz, K. M., Stanton, R. V., Cheng, A. L., Vincent, J. J., Crowley, M., Ferguson, D. M., Radmer, R. J., Seibel, G. L., Singh, U. C., Weiner, P. K. and Kolman, P. A. (1997) AMBER 5. *University of California, San Francisco*.

31. Gelin, B. R. and Karplus, M. (1979) Side-chain torsional potentials: effect of dipeptide, protein, and solvent environment. *Biochemistry*, **18**(7)**,** 1256-68.

32. Hunter, C. A. and Lu, X. J. (1997) DNA base-stacking interactions: A comparison of theoretical calculations with oligonucleotide X-ray crystal structures. *J. Mol. Biol.*, **265**(5)**,** 603-619.

33. Broyde, S. and Hingerty, B. E. (1987) Visualization of an AAF induced frameshift mutation: molecular views of base displacement in B-DNA from minimized potential energy calculations. *Nucleic Acids Res.*, **15**(16)**,** 6539-52.

34. Schmitz, U., Sethson, I., Egan, W. M. and James, T. L. (1992) Solution structure of a DNA octamer containing the Pribnow box via restrained molecular dynamics simulation with distance and torsion angle constraints derived from two-dimensional nuclear magnetic resonance spectral fitting. *J. Mol. Biol.*, **227**(2)**,** 510-31.

35. Kirpatrick, S. J., Gellat, C. D. and Vecchi, M. P. (1983) Optimization by simulated annealing. *Science*, **220,** 671-680.

36. Kozack, R. E. and Loechler, E. L. (1997) Molecular modeling of the conformational complexity of (plus)-anti-B(alpha)PDE-adducted DNA using simulated annealing. *Carcinogenesis (Oxford)*, **18**(8)**,** 1585-1593.

37. Kozack, R. E., Shukla, R. and Loechler, E. L. (1999) A hypothesis for what conformations of the major adduct of (+)-anti-B(a)PDE (N2-dG) causes GfwdarwT versus GfwdarwA mutations based upon a correlation between mutagenesis and molecular modeling results. *Carcinogenesis (Oxford)*, **20**(1)**,** 95-102.

38. Kozack, R. E. and Loechler, E. L. (1999) Molecular modeling of the major adduct of (+)-anti-B(a)PDE (N2-dG) in the eight conformations and the five DNA sequences most relevant to base substitution mutagenesis. *Carcinogenesis (Oxford)*, **20**(1)**,** 85-94.

39. Shapiro, R., Sidawi, D., Miao, Y. S., Hingerty, B. E., Schmidt, K. E., Moskowitz, J. and Broyde, S. (1994) Conformation of amine-modified DNA: 2-Aminofluorene- and 2-(acetylamino)fluorene-modified deoxydinucleoside monophosphates with all possible nearest

neighbors. A comparison of search and optimization methods. *Chem. Res. Toxicol.*, **7**(2)**,** 239-253.

40. Schmitz, M. and Steger, G. (1996) Description of RNA folding by "simulated annealing". *J. Mol. Biol.*, **255**(1)**,** 254-266.

41. Peyert, N., Seneviratne, A. P., Allawi, H. T. and SantaLucia, J. (1998) Nearest-Neighbor Thermodynamics and NMR of DNA sequences with Internal AA, CC, GG and TT Mismatches. *Biochemistry*, **38,** 3468-3477.

42. Burkard, M. E., Kierzek, R. and Turner, D. H. (1999) Thermodynamics of Unpiared Terminal Nucleotides on Short RNA Helixes Correlates with Stacking at Helix Termini in Larger RNAs. *J. Mol. Biol.*, **290,** 967-982.

43. Gibbs, E. J., Maurer, M. C., Zhang, J. H., Reiff, W. M., Hill, D. T., Malicka-Blaszkiewicz, M., McKinnie, R. E., Liu, H.-Q. and Pasternack, R. F. (1988) Interactions of Porphyrins with Purified DNA and More Highly Organized Structures. *J. Inorg. Biochem.*, **32,** 39-65.

44. Pasternack, R. F., Giannetto, A., Pagano, P. and Gibbs, E. J. (1991) Self assembly of porphyrins on nucleic acids and polypeptides. *J. Amer. Chem. Soc.*, **113,** 7799-7800.

45. Kelly, J. M., Murphy, M. J., McConnell, D. J. and OhUigin, C. (1985) A comparative study of the interaction of 5,10,15,20-tetrakis (N- methylpyridinium-4-yl)porphyrin and its zinc complex with DNA using fluorescence spectroscopy and topoisomerisation. *Nucleic Acids Res.*, **13**(1)**,** 167-84.

46. Banville, D. L., Marzilli, L. G., Strickland, J. A. and Wilson, W. D. (1986) Comparison of the effects of cationic porphyrins on DNA properties: influence of GC content of native and synthetic polymers. *Biopolymers*, **25**(10)**,** 1837-58.

47. Wuthrich, K. (1986) NMR of Proteins and Nucleic Acids. *NMR of Proteins and Nucleic Acids, J. Wiley and Sons, Inc.*

48. Massire, C. and Westhof, E. (1998) MANIP: an interactive tool for modelling RNA. *J Mol Graph Model*, **16**(4-6)**,** 197-205, 255-7.

49. Janson, T. R. and Katz, J. J. (1979) Nuclear Magnetic Resonance Spectroscopy of Diamagnetic Porphyrins. *The Porphyrins, Academic Press. Inc.*, **vol. IV,** 1-57.

FIGURE LEGENDS

Figure 1. A) The cationic porphyrin, meso-tetrakis (para-N-trimethylaniliniumyl) porphine (TMAP). B) DNA three-way junction (3WJ). Helical arms are labeled I, II and III. Arms II and III are colinearly stacked as shown by the arrow. Arrows indicate stacking interactions observed by NMR C) Minihairpin loop (MH DNA) used for gel electrophoresis experiments D) Minihairpin loop with the extended duplex (EX DNA) used for the optical absorption and CD studies.

Figure 2. Native gel mobility shift analysis of TMAP binding to the MH DNA and 3WJ DNA. The MH DNA samples stem loop (lines 1-5) and 3WJ (lines 6-10) at the same concentration were titrated with increasing amounts of TMAP as indicated (r_0 = [TMAP]/[DNA]). Panels A and B represent the same polyacrylamide gel photographed in two different ways. In panel A, DNA bands were visualized by UV shadowing of the gel on a fluorescent TLC plate using UV lamp (254 nm). In panel B the positions of porphyrins on the gel were visualized by fluorescence generated by UV transillumination of the gel. This figure shows specific TMAP binding by the 3WJ with high affinity.

Figure 3. A) UV titration of TMAP with 3WJ DNA at ratios of [DNA]/[P] equal to 0.0; 0.25; 0.5; 1.0 and 2.0 in 20 mM phosphate buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.05 M NaCl, 0.1 mM EDTA), pH=7.2 at room temperature. B) Circular dichroism measurements of TMAP with 3WJ DNA at ratios of [DNA]/[P] (R) equal 0.25; 0.5; 1.0; 2.0 in 20 mM phosphate buffer, pH 7.2, containing 0.05 M NaCl and 0.1 mM EDTA at room temperature.

Figure 4. A) UV titration of TMAP with EX DNA at ratios of [DNA]/[P] (R) equal 0.0; 0.25; 0.5; 1.0; 2.0, and 3.0 in 20 mM phosphate buffer, pH 7.2, containing 0.05 M NaCl and 0.1 mM EDTA at room temperature. B) Circular dichroism measurements of TMAP with EX DNA at ratios of [DNA]/[P] (R) equal 0.25; 0.5; 1.0; 2.0 in the same buffer.

Figure 5. A) 1D spectra of the 3WJ DNA (no TMAP in solution; B) 1D NMR spectra of the TMAP/3WJ complex at [P]/[DNA] ratio = 0.5; C) [P]/[DNA] = 1.0;
D) [P]/[DNA] = 2.0. The arrow indicates the resonance from the TMAP methyl groups. All spectra were acquired at 7°C.

Figure 6. A) Methyl region of the 1D spectrum of the DNA 3WJ in absence of TMAP; B) Methyl region of the 1D NMR spectra of the TMAP/3WJ complex at the [P]/[DNA] = 0.5; C) The same region for [P]/[DNA] = 1.0; D) The same region for [P]/[DNA] = 2.0. Signals caused by impurities are indicated with an asterisk (x). All spectra were acquired at 7°C to reduce chemical exchange between the bound and free drug

Figure 7. Aromatic region of 2D NOESY spectra of the DNA 3WJ and TMAP/3WJ complexes acquired at 7°C with 300 ms mixing time. A) DNA only. B) TMAP/3WJ complex at [TMAP]/[3WJ] = 0.5. C) TMAP/3WJ complex at [TMAP]/[3WJ] = 1.0. The assignments of porphyrin protons are shown.

Figure 8. A) Sequential assignments of aromatic, H1', and CH5 protons of junction and hairpin residues in the DNA 3WJ in absence of TMAP. The NOESY spectrum was acquired at 7°C with 300 ms mixing time. The dashed lines show crucial sequential assignments at the junction that

indicate the stacking of helix II on helix III (see Figure 1) and the solid lanes residues belonging to the hairpin loops. . The cytosine H6-H5 crosspeaks are labeled in bold font. Intra-residue aromatic-H1' crosspeaks are labeled by residue. Inter-residue aromatic-H1' crosspeaks between an aromatic proton and H1' of the preceding residue are labeled according to the residue of the aromatic proton and are distinguished with an asterisk (*). B) Sequential assignments of aromatic, H1', and CH5 protons of junction and hairpin residues in the DNA 3WJ in presence of TMAP ([TMAP]/[3WJ] = 0.5). The NOESY spectrum was acquired at 7°C with 300 ms mixing time. The dotted lines indicate junction nucleotides and the solid lanes residues belonging to the hairpin loops. The crosspeaks are labeled as described in Figure 8A and NOEs affected by the presence of the TMAP are in red.

Figure 9. The thymidine-methyl to aromatic region of the 300 ms NOESY spectrum of the DNA 3WJ acquired 7°C in H_2O at [TMAP]/[3WJ] = 0.5. The crosspeaks are labeled according to Figure 8B. Crucial NOEs between T28 and C7 are indicated with an arrow. This NOE proved stacking of helix I on helix II. The NOEs T13Me-C11H6 and T23Me-C21H6 indicate stacking between T13 and C11 and between T23 and C21.

Figure 10. TMAP/3WJ and TMAP/duplex complexes obtained by Simulated Annealing (duplex = $[d(CCCGGG)_2]$). DNA backbone is shown using cyan ribbon, which helps to visualize flexibility provided by the unpaired T28 and T29 nucleotides to 3WJ conformation to accommodate TMAP molecule (in yellow) at the junction site.

Table 1. Selective broadening of inter-residue NOEs in 3WJ DNA induced by binding of TMAP. "-" indicates that the peak is too broad to be observed or overlaps other peaks.

Motif	Base 1	Base 2	TMAP/3WJ = 0	TMAP/3WJ =0.5
Junction	T28	C7	T28H6-C7H2'1	Broadened
			T28H6-C7H2'2	Broadened
			T28Me-C7H5	Broadened
			T28Me-C7H6	Broadened
			T28Me-C7H3'	-
			T28Me-C7H1'	-
	T28	G27	T28Me-G27H3'	-
	G27	C8	G27H8-C8H5	-
			G27H1'-C8H6	Broadened
	C18	G17	C18H6-G17H1'	Broadened
			C18H5-G17H8	-
	G30	T29	G30H8-T29H4'	-
	T29	T28	T29Me-T28H6	Broadened
			T28Me-T29H6	Broadened
			T29H6-T28H1'	Broadened
			T29H6-T28H2'1	Broadened
			T29H6-T28H2'2	-
			T29H6-T28H3'	-
Hairpin loop	T23	C21	T23Me-C21H6	Unaffected
			T23Me-C21H1'	Unaffected
			T23Me-C21H5	Unaffected
	T13	C11	T13Me-C11H6	Unaffected
			T13Me-C11H1'	Unaffected
			T13Me-C11H5	Unaffected

Table 2. AMBER energies for the TMAP/3WJ, TMAP/duplex and T4MPyP/duplex complexes studied. Total AMBER energy is broken down by energy terms. The Interaction Energies (red) are calculated as the difference of the complex energies (black) and the sum of the porphyrin and DNA alone (blue).

A)

Energy term (kca/mol)	TMAP/3WJ complex	3WJ Alone	TMAP Alone	Interaction Energy	TMAP/ Duplex	Duplex Alone	TMAP Alone	Interaction Energy
AMBER TOTAL	-1560.1	-1371.7	171.4	-359.8	-672.0	-664.0	180.2	-188.2
BOND	47.6	43.4	4.2	0.0	23.1	17.6	5.5	0.0
VDWAALS	-690.7	-602.2	-1.1	-87.4	-183.0	-147.5	-3.6	-31.9
1-4 VDW	304.6	276.1	28.4	0.2	118.9	90.0	28.9	0.0
ANGLE	221.0	197.0	24.3	-0.3	94.9	67.2	27.7	0.0
EEL	-165.7	-274.5	377.7	-268.9	119.4	-103.2	378.0	-155.4
1-4 EEL	-2054.1	-1659.2	-394.4	-0.5	-1205.3	-807.9	-397.5	0.0
DIHED	777.2	645.8	132.3	-0.9	360.4	219.3	141.1	0.0

Energy term (kcal/mol)	T4MPyP/ Duplex	Duplex Alone	T4MPyP Alone	Interaction Energy
AMBER TOTAL	-962.0	-809.2	77.3	-230.1
BOND	19.3	17.9	1.3	0.0
VDWAALS	-284.0	-207.7	-1.3	-75.0
1-4 VDW	163.7	137.5	26.3	-0.1
ANGLE	91.0	75.4	15.4	0.3
EEL	-253.4	-264.7	166.6	-155.3
1-4 EEL	-1058.0	-841.2	-216.6	-0.2
DIHED	359.0	273.6	86.1	-0.7

Table 2 cont.

B)

Energy term (kcal/mol)	TMAP in 3WJ	TMAP minimized	Difference	TMAP in Duplex	Difference	Duplex T4 only	T4MPyP minimized	Difference
AMBER TOTAL	171.4	114.0	57.4	180.2	66.2	77.3	72.3	5.0
BOND	4.2	3.1	1.1	5.5	2.4	1.3	2.2	-0.9
VDWAALS	-1.1	-11.3	10.2	-3.6	7.7	-1.3	-2.1	0.8
1-4 VDW	28.4	18.9	9.5	28.9	10.0	26.3	24.0	2.3
ANGLE	24.3	20.6	3.7	27.7	7.1	15.4	15.7	-0.3
EEL	377.7	280.7	97.0	378.0	97.3	166.6	164.0	2.6
1-4 EEL	-394.4	-264.3	-130.1	-397.5	-133.2	-216.6	-213.0	-3.6
DIHED	132.3	66.7	65.6	141.1	74.4	86.1	81.0	5.1

C)

Energy term (kcal/mol)	TMAP Duplex	T4MPyP Duplex	Difference
AMBER TOTAL	-664.0	-809.2	145.2
BOND	17.6	17.9	-0.4
VDWAALS	-147.5	-207.7	60.2
1-4 VDW	90.0	137.5	-47.5
ANGLE	67.2	75.4	-8.2
EEL	-103.2	-264.7	161.4
1-4 EEL	-807.9	-841.2	33.4
DIHED	219.3	273.6	-54.3